Degradation of bisphenol A by different fungal laccases and identification of its degradation products

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Abstract

Different fungal laccases were used as biocatalysts for the biotransformation of bisphenol A (BPA). The quantitative analysis by gas chromatography-mass spectrometry (GC-MS) showed that BPA is more rapidly oxidized by *Coriolopsis gallica* laccase among the different fungal laccases tested. Carboxylic acid derivatives such as tartaric acid was found as BPA degradation products resulting from reactions catalyzed by 1 U ml^{-1} of laccase from *C. gallica* in the presence of 1mM of the laccase-mediator 1-hydroxybenzotriazole (HBT), while β-hydroxybutyric acid resulted from oxidative reactions without HBT. BPA was completely removed within 3 h and pyroglutamic acid was found as a supplementary oxidative degradation product from HBT when identified by GC-MS. Laccase played a critical role in BPA biodegradation and catalyzed a cross-coupling reaction.

Key words : Bisphenol A, Laccases, Biodegradation, Identification , Intermediates, pathway.

1. Introduction

Bisphenol A (BPA) is one of the most widely manufactured chemical in the world due to its broad spectrum of applications and growing demand. Around 95% of BPA is used for the production of synthetic polymers including epoxy resins and polycarbonates (Flint et al., 2012). The final products are used as coatings on cans, powder paints, additives in thermal paper, dental fillings and as antioxidants in plastics.

However, BPA has been identified as an Endocrine Disrupting Chemical (EDC) by the US Environmental Protection Agency (EPA) and the World Wide Fund for Nature (WWF) and has been declared as a social, environmental and global issue (Mohapatra et al., 2010). EDCs are synthetic chemicals that may interfere with the body 's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife (Chen et al., 2008; Muelle and Heger, 2013). The health effects from exposure to BPA have been investigated elsewhere (Sun et al., 2013).

Thus, during the last years, the degradation of BPA has been extensively studied. Advanced oxidation processes, such as Fenton reaction (Mohapatra et al., 2011), ozonation (Wang et al., 2014), photocatalytic oxidation (Kuo et al., 2014; Tan et al., 2015), ultrasonic oxidation (Yu et al., 2014) and combined techniques (Nie et al., 2014), can efficiently oxidize BPA owing to the production of hydroxyl radicals. However, these technologies present several important disadvantages since they are time-consuming methodologies, have high costs, and lead to toxic residues (Michałowicz, 2014).

Furthermore, several combined anaerobic and aerobic microbial treatments have been suggested to enhance the degradation of BPA using bacterial consortia (Eio et al., 2014; Kamaraj et al., 2014) and the acclimation strategy (Fischer et al., 2010). However, the acclimation process to a xenobiotic, such as BPA, could interfere with the normal biodegradable substrates (Ferro Orozco et al., 2013) and many bacterial metabolic pathways will be involved in the biodegradation process (Eio et al., 2014).

Ligninolytic fungi have been studied as a possible agent with extensive biodegradation properties for several pollutants because of the production of non-specific oxidative enzymes, i.e. manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) or laccases (Lac) (Reddy, 1995; Gianfreda et al., 2006).

Among these oxidative enzymes, laccases from the white rot fungus (WRF) have attracted more attention in treatment of EDCs at low concentrations due to its high reactivity and selectivity. Chairin et al. (2013) reported the efficiency of laccase from *Trametes polyzona* in the biodegradation of BPA. Moreover, studies on laccases of the basidiomycetes *Trametes villosa* (Fukuda et al., 2001), *Coriolopsis polyzona* (Cabana et al., 2007), *Trametes polyzona* (Chairin et al., 2013), *Trametes versicolor*, *Polyporus pinisitus* (Kim et al., 2008; Margot et al., 2013), and *Grifola frondosa* (Nitheranont et al., 2011), as well as laccases from other basidiomycetes (Tanaka et al., 2000; Kim and Nicell, 2006), revealed the potential of these oxidative enzymes for BPA degradation. Although laccases have lower redox potential, their expanded role on non-phenolic compounds, in the presence of a low molecular mass mediator, as well as their utilization of oxygen, a non-limited electron acceptor for their catalytic activity, makes them more suitable for industrial and environmental purposes (Ca ñas and Camarero, 2010; Camarero et al., 2014).

Many studies reported that laccase activity could be enhanced during the degradation of several recalcitrant chemicals such as 4-n-nonylphenol and aniline (Mougin et al., 2002), 2,5-xylidine (Kollmann et al., 2005; Garcia et al., 2006), bisphenol A and diclofenac (Yang et al., 2013; Nguyen et al., 2014) or nonylphenol (Kim et al., 2008)

In this paper we studied the potential of fungal laccases to degrade BPA, analyzing by gas chromatography-mass spectrometry (GC-MS) the biodegradation pattern of this compound.

Materials and Methods

2.1. Reagents

BPA [2,2-Bis-(4-hydroxyphenyl)propane]) (Cas: 80-05-7) of analytical grade, was purchased from Rankem, India. Silica (60-120 mesh) for the cleaning of samples, N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) for silylation and anhydrous sodium sulphate for drying solvent extracts prior to ga s chromatography (GC) analysis, were obtained from Sigma-Aldrich. Ethyl acetate was purchased from Merck.

1.2. Fungal strains

The fungal strains used in this study were *Coriolopsis gallica* (BS54) [KJ412304], *Bjerkandera adusta* (11B) [KU904462] and *Trametes versicolor* (A3) [KU904463] isolated in Tunisia. The strains were maintained on Petri plates containing 2% malt extract agar (MEA) at 4 °C and sub-cultured every 2 months.

2.3. Culture conditions

Fungal strains were cultured in semi-solid-state fermentation conditions in 250-ml cotton-plugged Erlenmeyer flasks containing 5.0 g of sawdust. The substrate was hydrated with 15 ml of minimal medium (MM), adjusting pH with 25 mM acetate buffer pH 5.0. This MM contained (g l^{-1}): glucose, 5; casein peptone, 6; KH_2PO_4, 0.025; $MgSO_4$ $7H_2O$, 0.25; KCl, 0.5. Inoculation was carried out directly in Erlenmeyer flasks. Six plugs (diameter 3 mm) from 5-day-old fungal cultures on malt extract agar (MEA) plates were added to each Erlenmeyer as inoculum. After fungal growth, the cultures were supplemented with 150 mM $CuSO_4$ sterile solution as laccase-inducer. The Erlenmeyer flasks were incubated statically for 12 days under an air atmosphere at 30 °C and 90% humidity, to avoid evaporation, in complete darkness. At the end of cultivation time the flasks content was extracted with sodium acetate buffer (pH 5.0, 10 mM) under shaking for 1 h, filtered and centrifuged at 7000 rpm for 20 min at 4 °C. The supernatant was collected and lyophilized to be used as biocatalyst for BPA degradation.

2.1. Enzyme activity assay

Laccase activity was measured using 10 mM 2,6-DMP in 100 mM sodium tartrate buffer, pH 5.0 by monitoring the absorbance at 469 nm using a 6705 JENWAY UV-Vis spectrophotometer (ε_{469nm} = 27500 M^{-1} cm^{-1}) (Muñoz et al., 1997).

2.2. Bisphenol A degradation

BPA (0.01%) was incubated in a reaction mixture containing 1.0 U ml^{-1} of the tested fungal laccases in 100 mM sodium acetate buffer (pH 5.0) at 45 °C under shaking. The enzymatic reaction was allowed to take place for 24 h, and then it was

stopped increasing the pH of the solution to 8.0 by adding sodium hydroxide. A BPA sample without added enzymes was treated simultaneously to be used as control. The reaction catalyzed by *C. gallica* (KJ412304) laccase in the presence or absence of a redox mediator, 1 mM hydroxybenzotriazole (HBT), was incubated for 3 h and the oxidative degradation products of bisphenol A were identified by GC-MS.

2.3. Extraction and derivatization

All samples were extracted twice with 2 volumes of ethyl acetate. The extracted solution was dehydrated with anhydrous sodium sulfate and concentrated by rotary evaporation. Triplicate aliquots of this solution were withdrawn and 5 µl of 0.01 % isotopically labeled BPA in the same solvent were added as internal standard, drying with a gentle nitrogen stream. Before GC-MS analysis, samples were trimethylsilylated (TMS) at 50 °C for 30 min using 0.2 ml of the BSTFA reagent.

2.4. BPA degradation products analysis by GC-MS

After derivatization, the low molecular weight compounds extracted from the BPA control sample and the laccase treatments were identified and quantified using a gas chromatograph equipped with a n HP-5MS column (30 m x 0.25 mm internal diameter; 0.25 µM film thickness) coupled to a quadrupole mass detector (GC-MS system 7980A-5975C, Agilent Technologies). Helium was the carrier gas at a flow rate of 1.2 ml min^{-1}. Injector and detector were programmed at 320 ºC. The column temperature was maintained at 50 °C for 1.5 min, increased to 150 °C at 10 °C min^{-1}, then to 300 °C at 20 °C min^{-1}, and held at 300 °C for 6 min. BPA and HBT were identified by comparison of their retention times and mass spectra with those of

commercial standards. Data were simultaneously acquired in full scan and SIM modes. Each full scan mass spectrum, recorded in the m/z range 33-800 amu (atomic mass units), was searched in the Wiley-NIST2011 database for compounds identification. SIM data were used for BPA quantification to determine the conversion rates. The SIM peak area from the five most abundant ions of the TMS ether derivative of BPA was referred to the area of the peak of ions m/z 73, 217, 368, 369, and 386 amu characteristic of the TMS ether derivative of the internal standard. Relying on this information, a calibration curve was constructed in the range of 0.25-2.5 μg ml^{-1}.

3. Results and discussion

3.1. Biodegradation of BPA by fungal laccases

The removal of BPA by different enzyme preparations from different fungal strains was performed at pH 5 and 45 °C. Fig. 1 shows the time course of BPA (0.01%) removal of in the presence of 1 U ml^{-1}laccase and 1 mM mediator.

After 1 h of treatment with *C. gallica* laccase, more than 62% of the BPA was removed from the solution. A decrease of 85% of BPA concentration was achieved after 2 h and 100% after 4 h. This reaction proceeded more slowly when catalyzed by laccases from the other strains. BPA removal after 1 h was lower than 50%, but 100% degradation occurred in all cases after 8 h.

In order to identify the BPA biodegradation products, assays with 1 U ml^{-1} of laccases from different fungal strains were carried out in Erlenmeyer flasks. Control samples without added enzymes and enzymatically treated (24 h) BPA samples were analyzed by GC-MS after extraction with ethyl acetate and derivatization (Fig. 2). In

review of the current methods for the BPA analytical determination in environmental, food and biological matrices, gas- or liquid-chromatography with mass spectrometric detection (GC-MS and LC-MS, respectively) would provide the best opportunity to obtain the desired detection limit and to identify the products from their mass spectra (Cabana et al., 2007; Han et al., 2015).

Since GC analysis of low volatility polar compounds, like phenolic and acidic compounds, results in poor sensitivity (Szyrwińska et al., 2007), derivatization methods have been extensively used including methylation, acetylation and silylation (Gatidou et al., 2007; Viñas and Campillo, 2014), which have frequently been resorted to the identification and quantification of BPA traces. Control reactions confirmed that BPA transformation was due to the laccase catalytic action. The chromatogram of the control sample showed the BPA peak, with retention time (R_t) of 14.4 min and the mass spectrum characteristic of this compound (Fig. 3). A second peak that eluted at 10.6 min, with a major ion of m/z 263 (Fig. 3), and that also appeared in the chromatograms of laccase-treated samples, may be considered as an undesirable BPA byproduct formed by the sodium hydroxide addition to stop the reactions. Oku et al. (2000) reported that the treatment of polycarbonate pellets (PC) in ethylene glycol (EG) with a catalytic amount of NaOH (0.1 equiv) produced BPA monohydroxyethyl ether. In our case, the database identified this product as 2,6-di-tert-butylphenol, which agrees with data reported by Verevkin (1999) suggesting that the degradation of alkylphenols may lead to the formation of tert-butylphenol.

The chromatograms depicted in Fig. 2 show that the BPA peak virtually disappeared after the laccase treatments for 24 h, while new peaks, corresponding to reaction products, were detected. When comparing the chromatograms of the

extracted compounds, a similar profile was observed in reactions catalyzed by laccases from the *B. adusta* and *T. versicolor* isolates (Fig. 2 a and 2 b), with one predominant product (Rt = 8.8 min) resulting from BPA oxidation. When the catalyst was the *C. gallica* enzyme, a major peak was detected at Rt = 7.0 min, indicating that the BPA oxidation mechanism of this laccase was not the same. Probably these laccases have different catalytic and kinetic properties, which deserve to be further studied

Among the laccases assayed in the present study, the one from *C. gallica* gave the highest BPA removal (91%) at the end of the oxidative reaction (24 h), as compared to those of *B. adusta* (85%) and *T. versicolor* (72%). These results agree with those reported by Cabana et al. (2009), who demonstrated that 3.75 U of immobilized laccases from *C. polyzona* remove BPA. A similar trend toward the biotransformation of BPA by fungal laccases was reported by many authors (Kim and Nicell , 2006; Cabana et al., 2007; Lloret et al., 2012).

After library-search of their full scan mass spectra in the Wiley-NIST2011 database, the final BPA degradation products were identified as glycerol for the laccases of *B. adusta* and *T. versicolor*, and β-hydroxybutyric acid, in the case of the reaction with *C. gallica* laccase. The mass spectra of the main peaks detected in the chromatograms are presented in Fig. 3.

3.2. Identification of the intermediates of BPA transformation by laccase/mediator system

Mediators are known to increase the substrate range of laccases (Kudanga et al., 2011). In this study, 1 mM of 1-hydroxybenzotriazole (HBT) was used as mediator and

tested to improve the BPA elimination.

Reactions (3 h) , catalyzed by *C. gallica* laccase, and control samples (without enzyme in the presence (+HBT) or absence of HBT (-HBT)) were analyzed by GC-MS (Fig. 4).

The profile in Fig. 4c shows a strong reduction of the BPA peak by action of 1 U ml^{1} of *C. gallica* laccase and the appearance of the other peaks that may correspond to BPA degradation products, since they are not present in the control chromatogram (Fig. 4a). Our data also show that the HBT peak (9.6 min) disappeared (Fig. 4d), indicating that this compounds is also oxidized and transformed.

In order to understand the BPA biodegradation process by *C. gallica* laccase, the identification of intermediate products originating from BPA degradation was attempted after GC-MS and library search, and the results are summarized in Table 1. Four intermediates with shorter retention time than BPA are gathered in Table 1. Some of those compounds have been identified as BPA degradation products such as carboxylic or polycarboxylic acids (tartaric acid and β-hydroxybutyric acid). However, hydrocinnamic acid and 3-phenyl-3-hydroxy propanoic acid are similar to the lignin metabolites (Pareek et al., 2001). So probably these compounds were from the culture medium and they were brought into the BPA-degradation system with fungal laccases.

Known intermediates that are more toxic than BPA but were not detected in this study are: *p*-hydroxyacetophenone, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, hydroquinone, and phenol (Ike et al., 2002; Lu et al., 2013**).** It is interesting to notice that all the identified compounds after the BPA biodegradation by laccase from *C.*

gallica are known as non toxic intermediates in industry of polycarbonate and waste plastics. According to Rameshwari and Meenakshisundaram (2013), β-hydroxybutyric acid is the precursor to polyesters, which are biodegradable plastics.

CG-MS data analysis revealed the accumulation of acidic oxidation intermediates. In a recent review of the BPA degradation products, Husain and Qayyum (2013) reported that the treatment of BPA by biological and enzymatic methods produced such polycarboxylic acids and other transformation products.

As mentioned above, the chromatogram of the reaction products from laccase reaction was markedly different following BPA treatment by the laccase/HBT system, since the mediator is also oxidized giving other degradation products and by-products. For example, tartaric acid detected upon this reaction, as well as a compound tentatively identified as pyroglutamic acid might have risen this from HBT degradation.

Based on the intermediate products identified here, a schematic view of the pathways for BPA degradation by *C. gallica* laccase was proposed (Fig. 5).

BPA oxidative cleavage results in the formation of β-hydroxybutyric acid in the absence of HBT, and of tartaric acid in the presence of HBT. The formation of tartaric and β-hydroxybutyric acids might be the result of the oxidation of the methyl groups on the propane moiety of BPA molecule under laccase action . Organic acids which commonly found as metabolic pathways intermediates are usually formed following ring fusion of aromatics (Spivack et al., 1994).

4. Conclusion

The experimental data suggest that bisphenol A is biodegradable by action of fungal

laccases.

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FIGURE CAPTIONS

Fig. 1 . Elimination of 0.01% of BPA with 1 U ml $^{-1}$laccase from (□) *C. gallica* , (Δ) *T. versicolor* and (•) *B. adusta* at pH 5.0 and 45 °C.

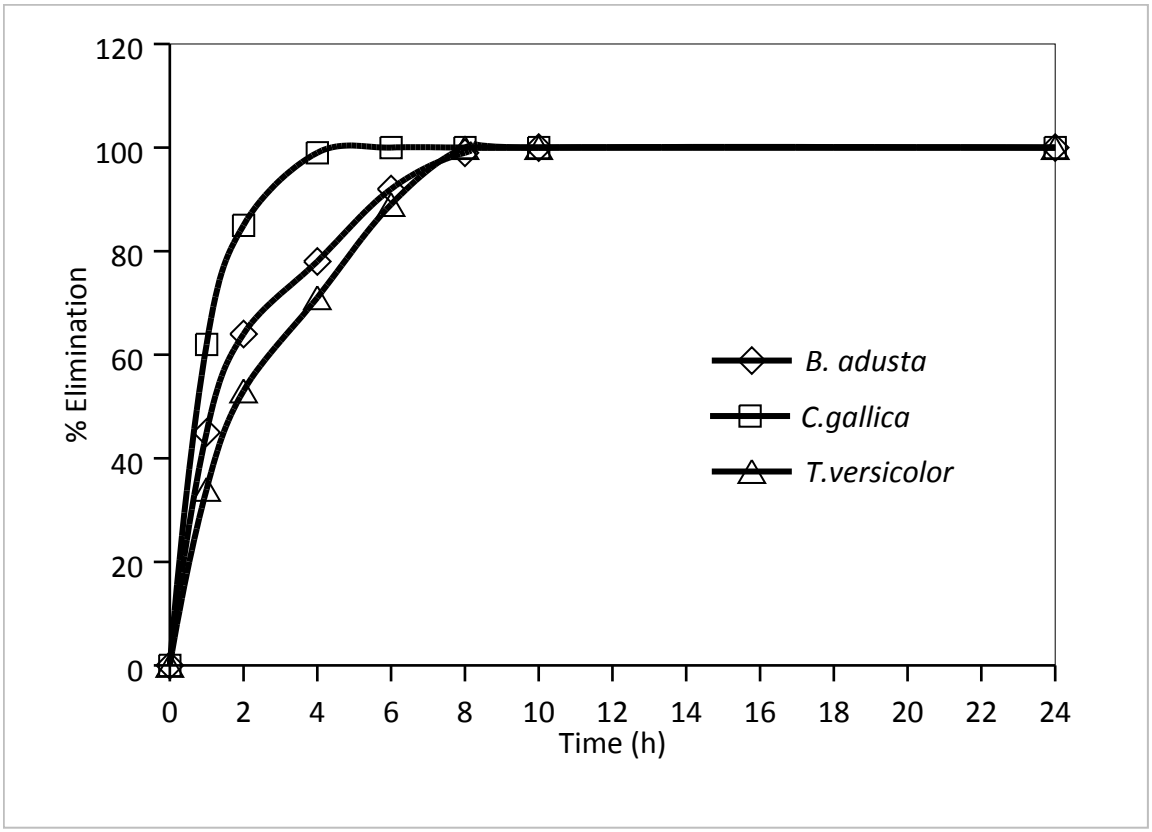
Fig. 2. GC-MS analysis of the compounds extracted with ethyl acetate from 24 h reactions of BPA with 1 U ml^{-1} of fungal laccases from: a) *B. adusta*; b) *T. versicolor*; c) *C. gallica* and d) Control sample of BPA without enzyme.

Fig. 3. Mass spectra of peaks at: a) Rt: 14.3 min (BPA), b) Rt: 10.6 min, attributed to a byproduct of BPA that may result from addition of sodium hydroxide, c) Rt: 8.1 min, the major product from treatment with laccases from *B. adusta* and *T. versicolor* glycerol, and d) Rt: 7.0 min, the main compound obtained by treatment with *C. gallica* laccase identified as β-hydroxy-butyric acid.

Fig. 4. Chromatograms showing the degradation of 0.01% of BPA. Negative controls: a) BPA without laccase and HBT, b) BPA and HBT without laccase. Reactions (3 h) catalyzed by 1 U ml^{-1} of *C. gallica* laccase: c) without HBT, and d) with 1 mM HBT.

Fig. 5. Pathways for the BPA degradation by *C. gallica* laccase.

Fig. 1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100
80
60
40
20
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% Elimination
0
2
4
6
8
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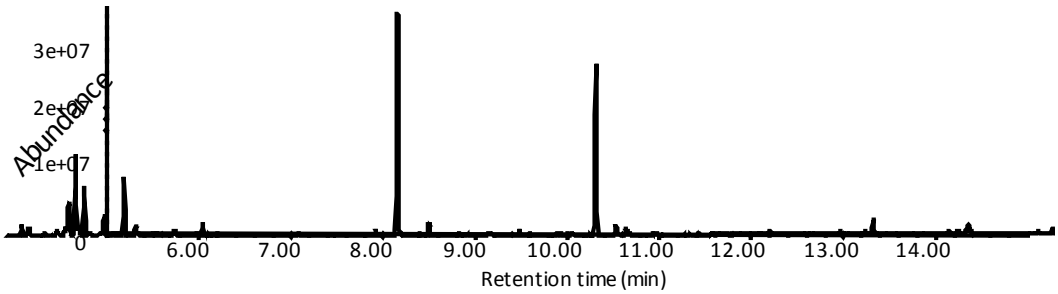
12
14
16
18
20
22
24
Time (h)
B. adusta
C.gallica
T.versicolor

Fig 2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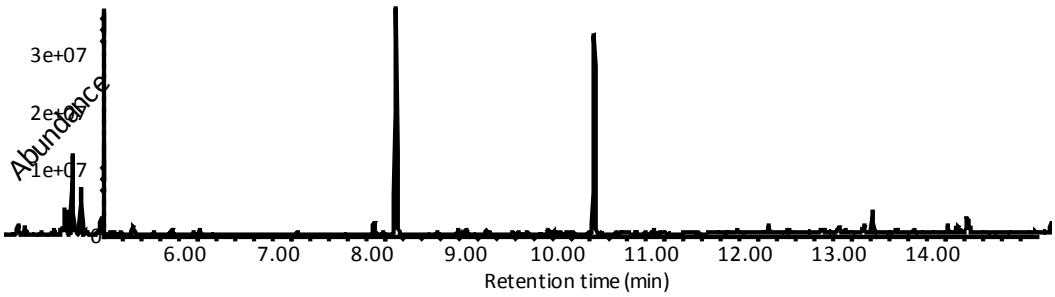
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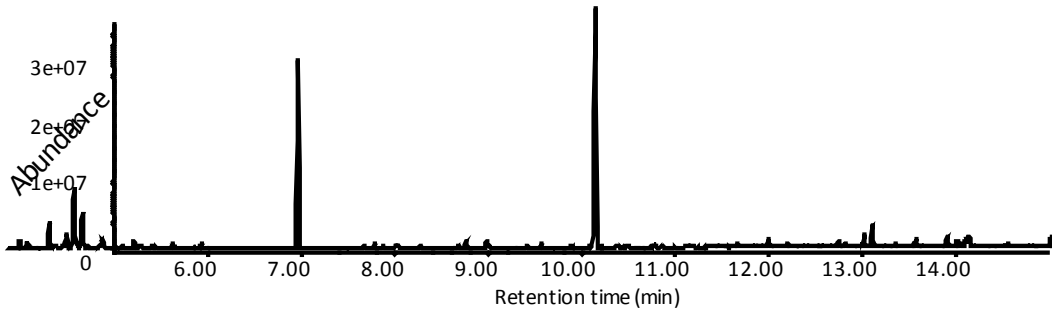
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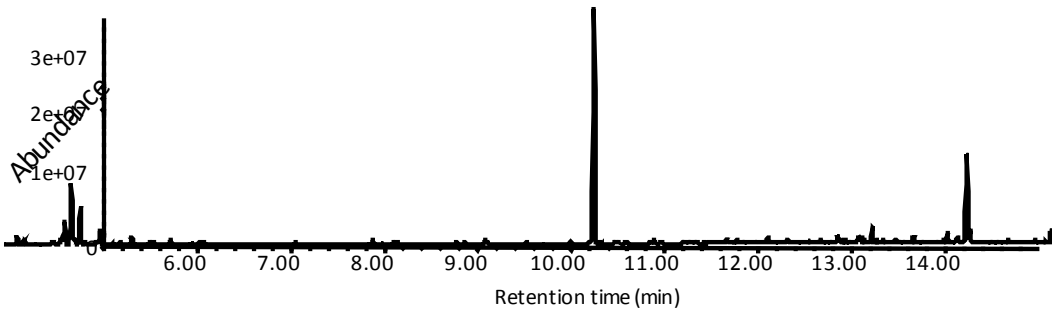
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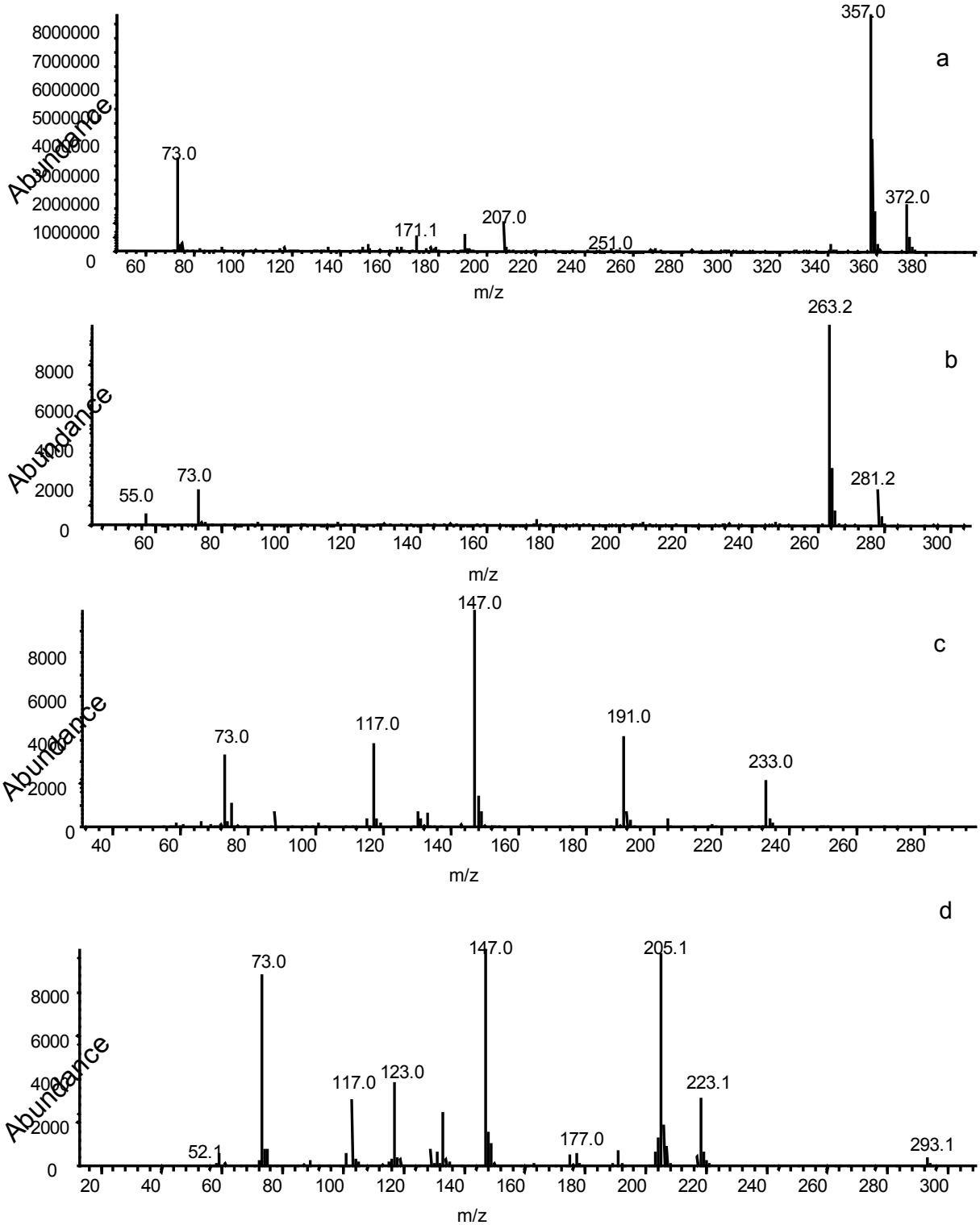
Fig 3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c
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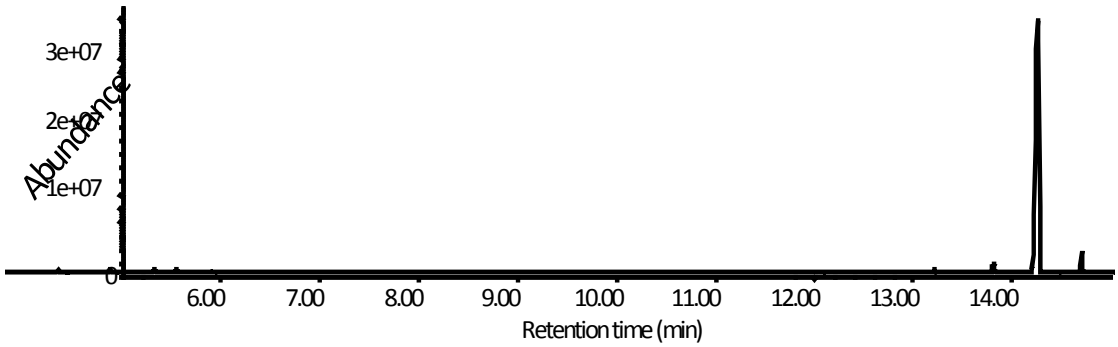
52.1
293.1

Fig 4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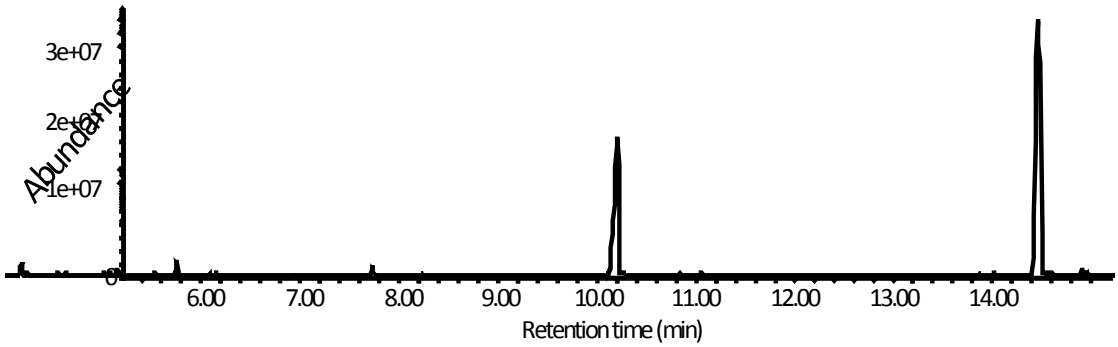
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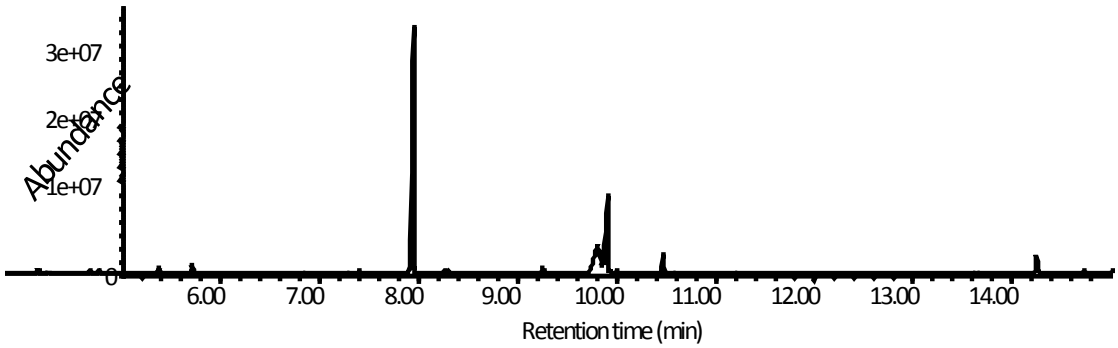
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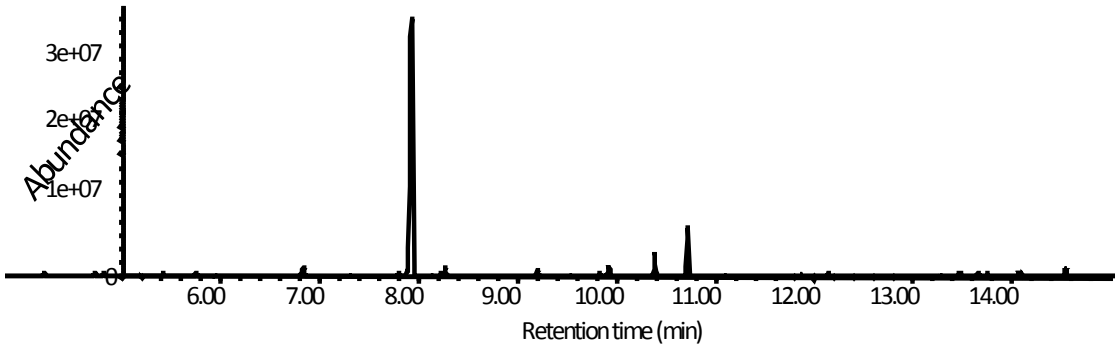
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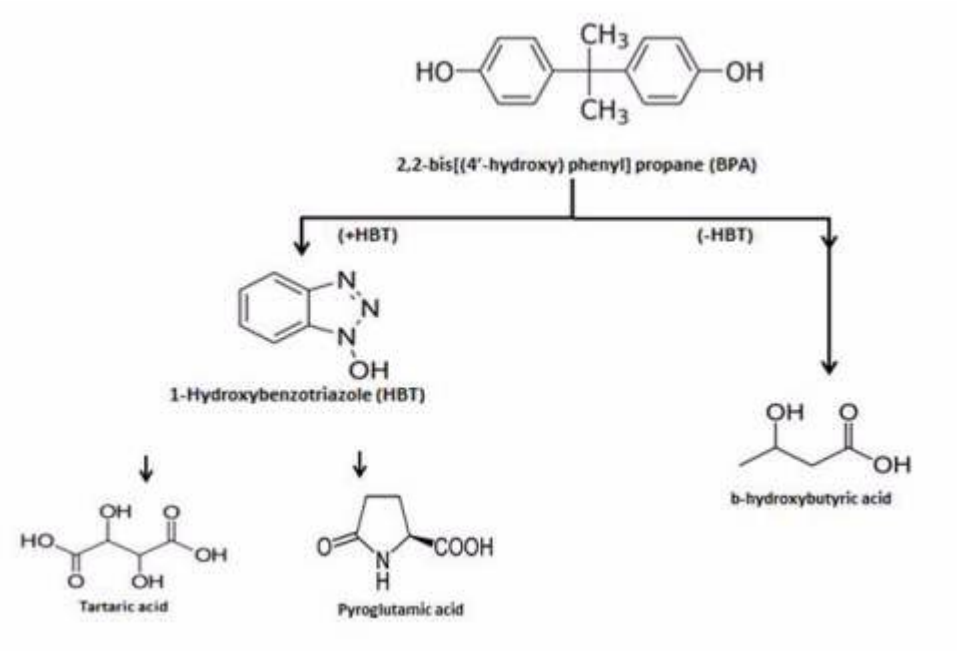
Retention time (min)

Fig 5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(+HBT)
(-HBT)
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N
N
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1-Hydroxybenzotriazole (HBT)
OH
O
OH
b-hydroxybutyric acid
HO
OH
O
O
OH
OH
Tartaric acid
O
N
H
COOH
Pyroglutamic acid